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Molecular Analysis in True Hermaphrodites With Different Karyotypes and Similar Phenotypes

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True hermaphroditism is characterized by the development of ovarian and testicular tissue in the same individual. Müllerian and Wolffian structures are usually present, and external genitalia are often ambiguous. The most frequent karyotype in these patients is 46,XX or various forms of mosaicism, whereas 46,XY is very rarely found. The phenotype in all these subjects is similar. We studied 10 true hermaphrodites. Six of them had a 46,XX chromosomal complement: 3 had been reared as males and 3 as females. The other 4 patients were mosaics: 3 were 46,XX/46,XY and one had a 46,XX/47,XXY karyotype. One of the 46,XX/46,XY mosaics was reared as a female, whereas the other 3 mosaics were reared as males. The sex of assignment in the 10 patients depended only on labio-scrotal differentiation. Molecular studies in 46,XX subjects documented the absence of Y centromeric sequences in all cases, arguing against hidden mosaicism. One patient presented Yp sequences (*ZFY*+, *SRY*+), which contrast with South African black 46,XX true hermaphrodites in whom no Y sequences were found. Molecular analysis in the subjects with mosaicism demonstrated the presence of Y centromeric and Yp sequences confirming the presence of a Y chromosome. Gonadal development, endocrine function, and phenotype in the 10 patients did not correlate with the presence of a Y chromosome or Y-derived sequences in the genome, confirming that true hermaphroditism is a heterogeneous condition. Both Mexican and non-South African 46,XX true hermaphrodites may be *SRY* positive.

KEY WORDS: *SRY*, true hermaphroditism, *TDF*, *ZFY*

INTRODUCTION

Male sex determination in mammals depends on the presence of a Y chromosome and the testicular determining factor (*TDF*) [Welshons and Russell, 1959; Page et al., 1987]. *TDF* was first located in the interval 1A1 on the Y chromosome, and the candidate gene was named *ZFY* [Page et al., 1987]. However, subsequently it was redefined in a 35 kb segment on the distal region of the short arm of the Y chromosome, proximal to the pseudoautosomal boundary. The gene found in this location is known as *SRY* (sex-determining region on Y chromosome) [Sinclair et al., 1990].

True hermaphroditism is a clinical condition in which ovarian and testicular tissues are present in the same individual, either in separate gonads or in one gonad (ovotestis) [van Niekerk and Retief, 1981]. These subjects usually present ambiguous external genitalia, whereas the development of Wolffian and Müllerian duct derivatives depends on the type of gonad and extent of testicular development in each case [Berkovitz et al., 1982; Pérez-Palacios et al., 1994].

The endocrine and gametogenic functions of the hermaphroditic ovary, either alone or in an ovotestis, appear to be normal in most patients [Tiltman and Sweerts, 1982; Pérez-Palacios et al., 1994]. However, the testis or the testicular portion of an ovotestis is usually abnormal in terms of hormone production and spermatogenesis [van Niekerk and Retief, 1981; Kofman-Alfaro et al., 1992].

Two-thirds of true hermaphrodites bear a 46,XX karyotype; the remainder have a 46,XX/46,XY or other mosaic karyotype. A 46,XY karyotype was also found in true hermaphrodites [van Niekerk, 1976; van Niekerk

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and Retief, 1981; Kofman-Alfaro et al., 1992; Pérez-Palacios et al., 1994]. Chimerism between fraternal fused twins or mosaicism following nondysjunction can explain the existence of true hermaphrodites with 46,XX and 46,XY cell lines [Jacobs, 1969; Berkovitz et al., 1982]. However, the genetic mechanisms responsible for 46,XX true hermaphroditism remain elusive. It is well known that in some cases, the entity can be explained because of the presence of an undetected cell line bearing a Y chromosome. In other cases, a translocation of chromosomal material encoding *TDF* from the Y to the X chromosome, or an autosomal or X-linked dominant mutation that permits testicular determination in the absence of *TDF*, have been proposed [Berkovitz et al., 1992].

Here we report the molecular findings (presence or absence of Y centromeric sequences, *ZFY* and *SRY*) in 10 true hermaphrodites and correlate them with the clinical, cytogenetic, and endocrinological data.

MATERIALS AND METHODS

Patients

Ten individuals with true hermaphroditism were studied. All were referred because of ambiguous external genitalia of variable degree. None of the patients had a family history related to the pathological entity, all being sporadic cases. Ages ranged from 10/12 to 27 years. Seven were <18 years and one of these was pubertal and two were postpubertal; all 3 of these patients had gynecomastia. In six patients, the sex of rearing had been masculine. All had a Mexican mestizo ethnic origin. External and internal genitalia, specific gonadal tissue, as well as other clinical traits are shown in Table I.

Cytogenetic Studies

Chromosome analysis was performed on peripheral blood leukocytes using GTG and CBG banding. In each patient, 100 metaphases were analyzed.

Endocrinological Studies

Measurement of baseline levels of immunoreactive serum gonadotropins, 17β-estradiol (E2), and testosterone (T) were made as previously described [Ulloa-Aguirre et al., 1988a,b]. In order to determine testosterone synthesis and secretion, an HCG stimulation test was performed. A total of 2,500 units (>4 years old) or 1,500 units of HCG (Gonadotropyl C, Roussel, México) were administered to each patient every 24 h for 4 consecutive days. Serum testosterone was measured before, during, and after gonadal stimulation.

Molecular Studies

DNA isolation. Genomic DNA was prepared from peripheral blood leukocytes by standard techniques [Sambrook et al., 1989].

Southern blot analysis. In all subjects and in female and male controls, 10 μg of genomic DNA were digested with *Eco*RI (Boehringer Mannheim GmbH, Mannheim, Germany). The digested DNAs were electrophoresed on 0.8% agarose gels and transferred to Duralose UV membranes (Stratagene, La Jolla, CA). The membranes were prehybridized by standard techniques [Sambrook et al., 1989] for 16 h at 42°C before the addition of radiolabeled α^{32}P dATP probe. Filters were hybridized at 48°C for probe pY97 and at 42°C for probe pDP1007. After hybridization, filters were washed with 0.2% SSC/0.1% SDS/50°C and subjected to autoradiography for several days at −70°C to X-OMAT K-film (Kodak, Guadalajara, Jalisco) as previously described [López et al., 1995].

DNA probes. Two probes were used to analyze the genomic DNA of the patients and normal controls: probe pY97 recognizes a Y-centromeric alphoid region [Wolfe et al., 1985]; and probe pDP1007 hybridizes with *ZFY* and *ZFX* genes [Page et al., 1987].

Polymerase chain reaction (PCR) analysis. Genomic DNA (500 ng) of patients and normal female and male controls were used. PCR was performed using the following oligonucleotides: Y1: 5′-ATGATAGAAACG-GAAATATG-3′; Y2: 5′-AGTAGAATGCAAAGGGCTCC-3′ which amplifies an alphoid repeat fragment of 170 bp from the Y-centromere. The PCR protocol has been previously described [Witt and Erickson, 1989, 1991].

PCR conditions for TDF4-TDF5 and SRY1-SRY2 were followed according to the method described by Bailey et al. (1992). TDF4: 5′-ATGTGGATGTCCA-CAAAGGT-3′ and TDF5: 5′-AAGCTTGTAGACAC-ACTGTT-3′, which flank a 400 bp region of *ZFY* [Page et al., 1987; Schneider-Gädicke et al., 1989]. SRY1: 5′-CGACAATGCAATCATATGC-3′ and SRY2: 5′-TAGCG-GTCCCGTTGCTGC-3′ [Sinclair et al., 1990], which flank a 609 bp fragment.

An aliquot of the amplified products was size-fractioned by electrophoresis through a 1.5% agarose gel containing ethidium bromide (0.0002%) and directly visualized by UV fluorescence. One-hundred bp ladder (BRL) was used as molecular weight size standard.

RESULTS

In cases 1 through 6, a 46,XX karyotype was documented. Cases 7 through 9 had a 46,XX/46,XY chromosomal complement, and patient 10 had a 46,XX/47,XXY karyotype. No autosomal structural abnormalities were found in any case and the sex chromosomes appeared normal (Table I).

An ovotestis was present in 7 patients and was bilateral in 4 of them. An ovary and ovotestis were present in 3 patients, a testis and an ovary in 2, and a testis and an ovotestis in one. In the postpubertal patients, the testis, as well as the testicular portion of the ovotestis, had Leydig cell hyperplasia, hypoplasia of the seminiferous tubules, and absence of germ cells. In the prepubertal individuals, immature gonads were observed. In postpubertal cases, both the ovary and the ovarian portion of the ovotestis were histologically normal. Internal genitalia varied depending on the adjacent existing gonad, although in case 1 the ovary was accompanied by derivatives of both Müllerian and Wolffian ducts. In all cases, uterus was found (Table I).

Prepubertal patients (cases 3, 4, 6–9) exhibited normal concentrations of gonadotropins according to their age, whereas the pubertal case (2) showed levels of go-

TABLE I. Description of 10 True Hermaphrodites Studied

Case	Sex of rearing	Age (years)	Phallus length (cm)	External genitalia (hypospadias)	Palpable gonads	Internal genitalia[a]		Karyotype (pctg)	Y		
						Right	Left		Cen	*ZFY*	*SRY*
1	M	27	4.8	Labioscrotal fusion (perineoescrotal)	Nonpalpable	Ovary, fallopian tube, Wollfian duct	Ovotestis, fallopian tube, Wollfian duct	46,XX (100)	–	+	+
2	M	10	3.1	Bifid scrotum (penile)	Right 1.8 ml	Testis, epididymis	Ovotestis, fallopian tube, epididymis, vas deferens	46,XX (100)	–	–	–
3	F	10/12	3.5	Labioscrotal fusion (perineoscrotal)	Nonpalpable	Ovotestis, fallopian tube, epididymis	Ovotestis, fallopian tube, epididymis	46,XX (100)	–	–	–
4	F	11/12	2.8	Labioscrotal fusion (perineoscrotal)	Nonpalpable	Ovotestis, fallopian tube	Ovotestis, fallopian tube	46,XX (100)	–	–	–
5	F	17	3.0	Labioscrotal fusion (perineoscrotal)	Nonpalpable	Ovary, fallopian tube	Testis, epididymis	46,XX (100)	–	–	–
6	M	4	2.8	Bifid scrotum (penoscrotal)	Left 1.7 ml	Ovary, fallopian tube	Testis, epididymis	46,XX (100)	–	–	–
7	M	2	2.8	Bifid scrotum (penoscrotal)	Nonpalpable	Ovary, fallopian tube	Ovotestis, fallopian tube	46,XX/46,XY (70/25)	+	+	+
8	M	2	2.0	Bifid scrotum (perineal)	Right 1.5 ml	Ovotestis, fallopian tube, epididymis	Ovotestis, fallopian tube epididymis	46,XX/46,XY (68/32)	+	+	+
9	F	1	3.0	Labioscrotal scrotum (perineal)	Nonpalpable	Ovotestis, fallopian tube	Ovotestis, fallopian tube	46,XX/46,XY (23/77)	+	+	+
10	M	16	3.5	Bifid scrotum (penile)	Nonpalpable	Ovotestis, fallopian tube	Ovary, fallopian tube	46,XX/47,XXY (72/28)	+	+	+

[a] All patients had a uterus.

nadotropins consistent with pubertal initiation. Both postpubertal individuals (cases 1 and 10) showed a discrete hypergonadotropism. In the pubertal and postpubertal patients, basal testosterone synthesis, as well as the maximal response to HCG were extremely variable, but concentrations were always below the existing normal values in control subjects. However, prepubertal subjects had age-appropriate basal testosterone concentration, and all cases responded, although in a variable degree, to the HCG challenge (Table II). In patient 1, who complained of cyclic hematuria, ovulation was documented by measuring progesterone concentrations every day for a month. Levels ranged from 4.3–11.0 ng/ml during the luteal phase.

Molecular studies revealed that none of the 46,XX patients had Y centromeric sequences as observed by DNA-DNA hybridization and PCR analysis (Table I), whereas the remaining subjects had a positive signal for Y centromeric sequences (Fig. 1, Table I). Cases 1 and 7–10 were positive for *ZFY* and *ZFX*, whereas the remaining subjects only had a positive hybridization signal for *ZFX* (Fig. 2, Table I). Fig. 2 shows the results of PCR amplification for a 400 bp fragment of *ZFY*. A positive band was observed in cases 1, and 7–10. The results of PCR amplification of the 609 bp *SRY* fragment are depicted in Figure 3. Only cases 1 and 7–10 exhibited a positive band of the expected size.

DISCUSSION

True hermaphroditism is a genetically heterogeneous condition with several possible courses. In 46,XX true hermaphroditism, 3 mechanisms have been proposed to explain testis determination: (1) hidden mosaicism with a Y-bearing cell line [Miró et al., 1978; Berkovitz et al., 1992], (2) translocation of Y material, including the *SRY* gene, from paternal Y to the X chromosome [Therkelsen, 1964; Ferguson-Smith, 1966], and (3) an autosomal or X-linked mutation that permits testis determination in the absence of *SRY* [Petit et al., 1987; Berkovitz et al., 1992; Fechner et al., 1993]. None of our 46,XX cases showed Y-centromeric sequences arguing against a hidden mosaicism. Furthermore, in cases 1, 5, and 6, mosaicism was excluded by PCR analysis in gonadal tissue (data not shown).

Molecular studies have demonstrated that 80% of XX males have Y material, including *TDF* [Affara et al., 1986; Palmer et al., 1989; Abbas et al., 1990; Numabe et al., 1992; Fechner et al., 1993]. On the contrary, only a few 46,XX true hermaphrodites showed detectable Y chromosome sequences, most of them being Y negative [Vergnaud et al., 1986; de la Chapelle, 1987; Page et al., 1987; Ramsay et al., 1988; Abbas et al., 1990; Nakagome et al., 1991; Pereira et al., 1991; Berkovitz et al., 1992; McElreavey et al., 1992; Kuhnle et al., 1993; Boucekkine et al., 1994]. Only 1 of our 6 46,XX true hermaphrodites (case 1) had Yp chromosomal sequences (*ZFY* and *SRY*). These results agree with previously reported studies and contrast only with South African blacks 46,XX true hermaphrodites where no Y material has been found [Spurdle et al., 1995].

Our 5 46,XX true hermaphrodites (*ZFY*−, *SRY*−) resemble Y-negative XX males, subjects in whom genital ambiguity and the absence of Y sequences are almost constant findings [Abbas et al., 1990; Pereira et al., 1991; Kuhnle et al., 1993]. Both 46,XX males and 46,XX true hermaphrodites may be alternative expressions of the same genetic mechanism, a hypothesis also supported by the occurrence of these two entities in the same pedigree [Berger et al., 1970; Skordis et al., 1987; Abbas et al., 1990; Kuhnle et al., 1993]. In these cases, testicular differentiation occurs in the absence of *SRY* and presumably results from a mutation of an autosomal or X-linked gene(s) that could act upstream or downstream of *SRY* [McElreavey et al., 1993]. Evidence from studies in patients with XY gonadal dysgenesis demonstrate the existence of two autosomal genes related to testicular differentiation: *WT1* is associated with 46,XY gonadal dysgenesis in Denys-Drash syndrome [Baird et al., 1992]; and the gene responsible for gonadal dysgenesis in campomelic dwarfism [Foster

TABLE II. Baseline Serum Levels of Gonadotropins and Testosterone, Plus Maximal Response (MR) to Exogenous HCG in Patients With True Hermaphroditism*

Patient	LH[a] (mIU/ml)	FSH[a] (mIU/ml)	Basal T (ng/ml)	T(MR) (ng/ml)	E2 (pg/ml)
1	13.3	3.5	1.32	4.16	60.0
2	3.7	2.8	0.36	0.73	<13
3	1.1	0.4	0.62	2.10	<13
4	0.8	0.3	0.03	1.54	<13
5	NM	NM	NM	NM	NM
6	ND	ND	0.42	1.73	28.0
7	1.0	0.4	0.20	0.45	<13
8	1.5	0.5	0.24	2.16	<13
9	ND	ND	0.93	4.91	13.0
10	12.1	5.3	1.42	3.93	37.0
Controls[b]					
Prepubertal	<3	0.5	<3.9[c]	0.6–6.8[c]	<60[d]
Postpubertal	3–12	0.5–5.0	3.9–10[c]	5.8–24.3[c]	<60[d]

*NM: not measured. ND: not detectable.
[a] Mean of two basal values.
[b] n = 60 [30 (3–10 years old), 30 (18–50 years old)].
[c] Male controls.
[d] Male controls and women during follicular phase.

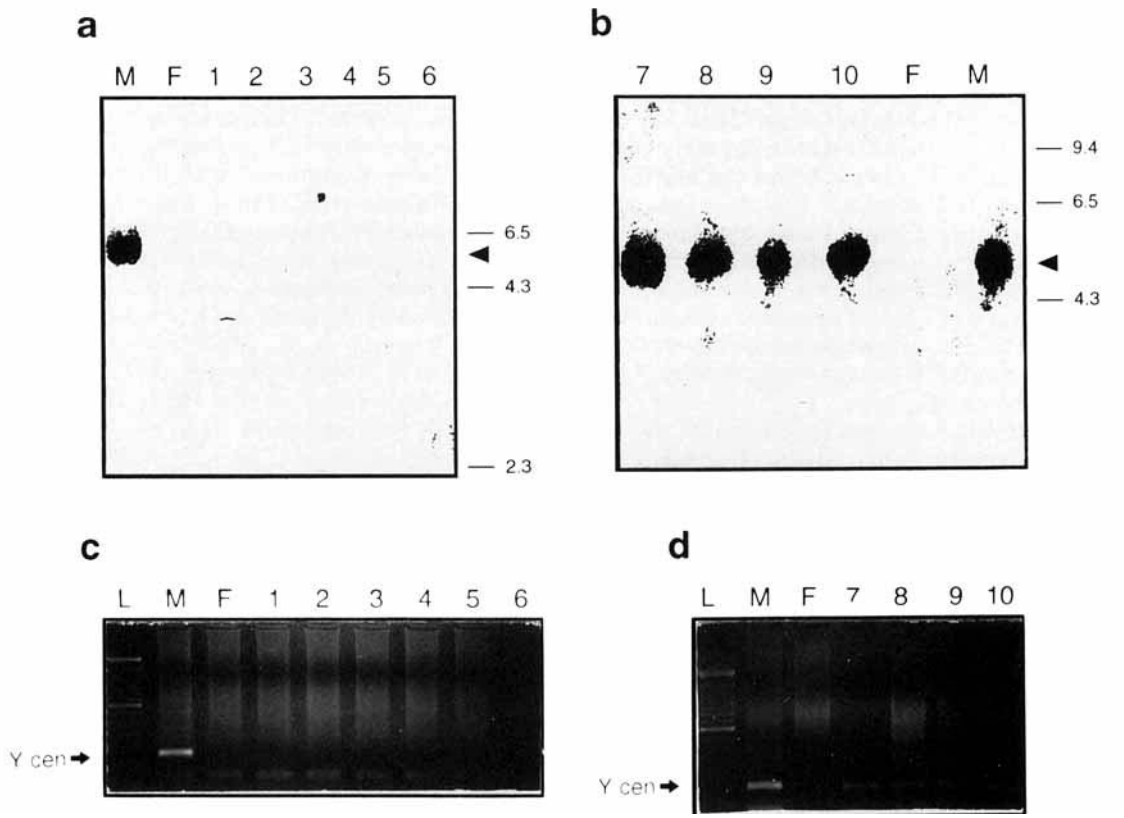

Fig. 1. Lanes M and F: male and female controls, respectively. Lanes 1–6: 46,XX true hermaphrodites. Lanes 7–10: true hermaphrodites with mosaic karyotypes. Lane L: 100 bp ladder (BRL). **(a,b):** Southern blot analysis using probe pY97. The *5.5* kb band is centromeric Y-specific region (◀). **(c,d):** Electrophoretic analysis of PCR products with Y1-Y2 primers. The presence of the Y-centromeric region is indicated by a 170 bp band.

et al., 1994; Wagner et al., 1994]. Another gene, *MIS*, would seem to be a candidate for a target gene immediately downstream of *SRY* [Haqq et al., 1994]. The deletion of 9p24 also has been related with genital ambiguity in the presence of *SRY* [Bennett et al., 1993].

To explain the presence of ovarian tissue in case 1 (*ZFY*+, *SRY*+), partial inactivation of the X chromosome carrying the translocated Y fragment may be the explanation for the heterogeneity of *SRY* expression in gonadal cells [Berkovitz et al., 1992; Fechner et al., 1994]. Another possibility could be that the amount of Y material or its specific location on the X chromosome may influence *SRY* expression, resulting in ovarian development [Berkovitz et al., 1992; McElreavey et al., 1992].

Hormonal levels in our patients showed that postpubertal subjects exhibited hypergonadotropic hypogonadism. Testosterone response after HCG stimulation, although apparently significant (due to the low basal levels), was always below the expected absolute concentrations for normal males. These results are on line with the histopathological findings of the testicular tissue, where Leydig cell hyperplasia, hypoplasia of the seminiferous tubules, and absence of germ cells were observed. On the contrary, in all children studied, gonadotropins, basal testosterone levels, and the testosterone response to HCG were within reference values for age. As in other pathological entities, notably the XX male, testicular damage is age-dependent and becomes more evident after puberty [Roe and Alfi, 1977; Kofman-Alfaro et al., 1985].

In the 6 46,XX true hermaphrodites, we attempted to correlate the sex of rearing, hormone levels, and histological characteristics with the absence or presence of Y-derived material. We did not find any clear relationship among these values, possibly because the only subject without a Y chromosome whose DNA contained *ZFY* and *SRY* had the smallest fragment of testicular tissue. In this patient the phallus length was bigger than in the other cases, but he was not more virilized than the *ZFY* and *SRY* negative individuals. However, this could be due to the adult age of the patient.

The reason for the presence of both gonadal tissues in our 3 46,XX/46,XY and one 46,XX/47,XXY true hermaphrodites must be different, at least in part, from those without mosaicism. In these cases, testis differentiation could be the consequence of the Y chromosome, whereas the existence of an ovary is likely to be due to the relative number of 46,XX cells [Berkovitz et al., 1982]. The study of chimaeras and mosaics, com-

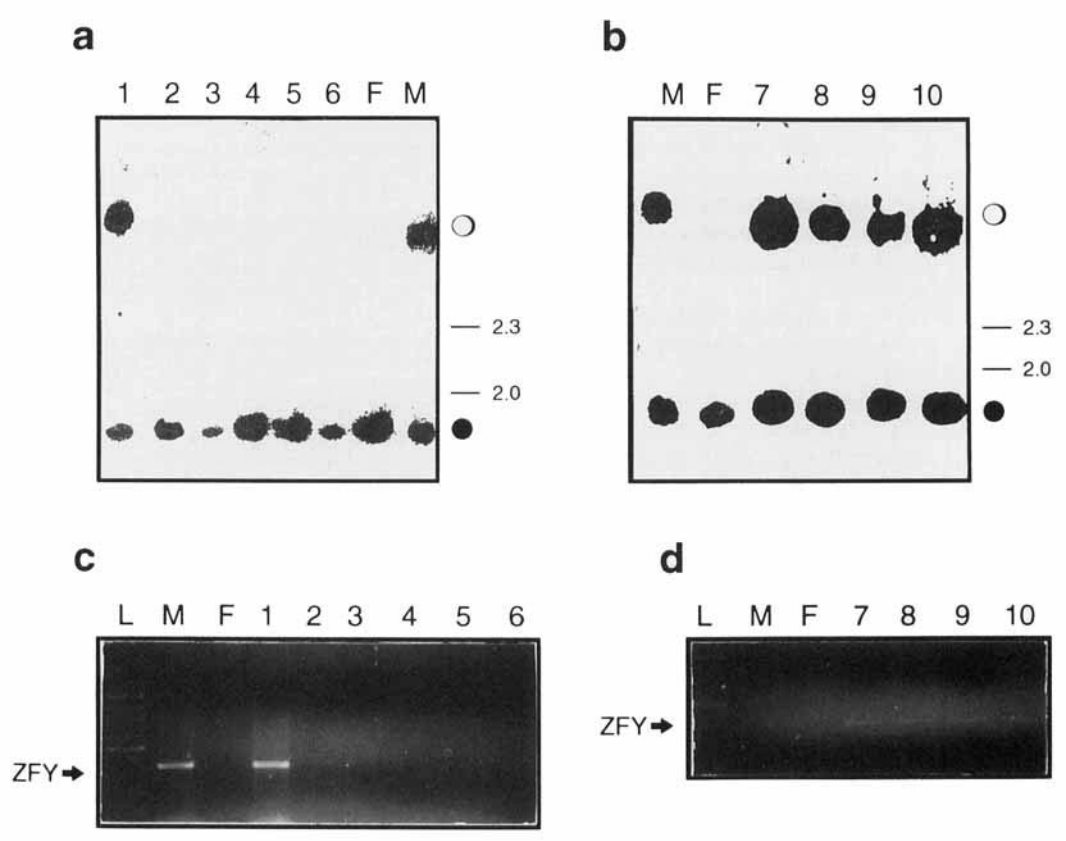

Fig. 2. Lanes M and F: male and female controls, respectively. Lanes 1–6: 46,XX true hermaphrodites. Lanes 7–10: true hermaphrodites with mosaic karyotypes. Lane L: 100 bp ladder (BRL). **(a,b):** Southern blot analysis using probe pDP1007. The *ZFX* 1.8 kb band (●) is X-specific; the *ZFY* 3.5 kb band (○) is Y-specific. **(c,d):** Electrophoretic analysis of PCR products amplified using *ZFY* primers. The presence of the *ZFY* gene is indicated by a 400 bp band.

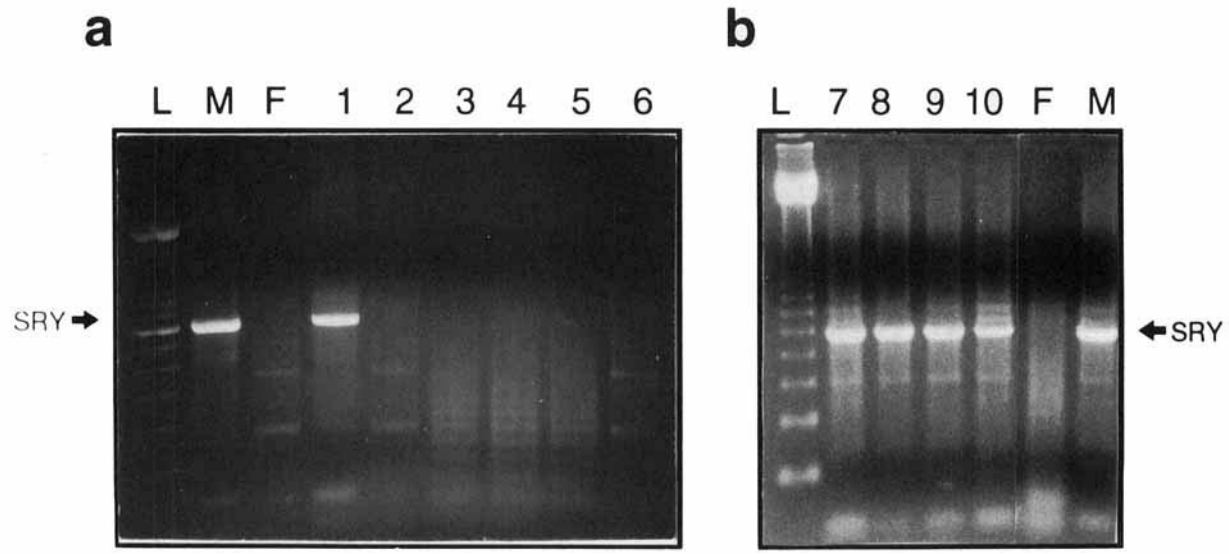

Fig. 3. Electrophoretic analysis of PCR products using *SRY* specific primers. The 609 bp amplified band indicates the presence of *SRY*. Lane L: ladder 100 bp (BRL). Lanes M and F: male and female controls, respectively. **(a)** Lanes 1–6: 46,XX true hermaphrodites. **(b)** Lanes 7–10: true hermaphrodites with mosaic karyotypes.

posed of XX and XY cells, indicates that for a testis to develop, a critical proportion of somatic cells in the genital ridge has to be XY [McLaren, 1984]. If the proportion of XY cells is too low, an ovary or occasionally an ovotestis develops. The critical cell type is probably the supporting cell precursor. Sertoli cells are almost exclusively XY, indicating that the testis-determining gene must act cell-autonomously within the supporting cell precursors to divert their fate along the Sertoli cell pathway [Lovell-Badge, 1992].

Our 4 mosaic patients have an apparently normal Y chromosome; 3 were reared as males, whereas one had a female sex assignment. In all of them, molecular studies showed the presence of Y centromeric and Yp sequence (*ZFY* and *SRY*), without a correlation between the relative number of cells that contain a Y chromosome and the type of gonad present.

The clinical, endocrinological, and histological traits among 46,XX and mosaic subjects were not characteristically different; the different sex assignment was due only to the degree of labio-scrotal differentiation.

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